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Nucleotide Sequence of the Lecithinase Operon of *Listeria monocytogenes* and Possible Role of Lecithinase in Cell-to-Cell Spread

JOSÉ-ANTONIO VAZQUEZ-BOLAND,[1]† CHRISTINE KOCKS,[1] SHAYNOOR DRAMSI,[1] HÉLÈNE OHAYON,[2] CHRISTIANE GEOFFROY,[3] JÉRÔME MENGAUD,[1] AND PASCALE COSSART[1]*

Unité de Génie Microbiologique et Laboratoire de Génétique Moléculaire des Listeria,[1] Station Centrale de Microscopie Eléctronique,[2] and Unité des Antigènes Bactériens,[3] Institut Pasteur, 25 Rue du Docteur Roux, Paris 75015, France

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The lecithinase gene of the intracellular pathogen *Listeria monocytogenes*, *plcB*, was identified in a 5,648-bp DNA fragment which expressed lecithinase activity when cloned into *Escherichia coli*. This fragment is located immediately downstream of the previously identified gene *mpl* (*prtA*). It contains five open reading frames, named *actA*, *plcB*, and ORFX, -Y, and -Z, which, together with *mpl*, form an operon, since a 5.7-kb-long transcript originates from a promoter located upstream of *mpl* (J. Mengaud, C. Geoffroy, and P. Cossart, Infect. Immun. 59:1043–1049, 1991). A second promoter was detected in front of *actA* which encodes a putative membrane protein containing a region of internal repeats. *plcB* encodes the lecithinase, a predicted 289-amino-acid protein homologous to the phosphatidylcholine-specific phospholipases C of *Bacillus cereus* and *Clostridium perfringens* (alpha-toxin). *plcB* mutants produce only small plaques on fibroblast monolayers, and an electron microscopic analysis of infected macrophages suggests that lecithinase is involved in the lysis of the two-membrane vacuoles that surround the bacteria after cell-to-cell spread. On the opposite DNA strand, downstream of the operon, three more open reading frames, *ldh*, ORFA, and ORFB, were found. The deduced amino acid sequence of the first one is homologous to lactate dehydrogenases. Low-stringency Southern hybridization experiments suggest that these three open reading frames lie outside of the *L. monocytogenes* virulence region: *mpl* and *actA* were specific for *L. monocytogenes*, sequences hybridizing to *plcB* were detected in *L. ivanovii* and *L. seeligeri*, and sequences hybridizing to ORFX, -Y, and -Z were found in *L. innocua*. In contrast to this, sequences hybridizing to *ldh* or ORFB were detected in all *Listeria* species (including the nonpathogenic ones).

Listeria monocytogenes is an aerobic, nonsporulating, gram-positive bacillus widespread in nature and responsible for human and animal listeriosis. The disease occurs in the form of severe opportunistic infections, i.e., abortions, meningitis and/or encephalitis, and septicemias, with a high case fatality rate in newborns and in adults whose defense mechanisms are impaired by pregnancy, therapeutically induced immunosuppression, underlying disease, or elderliness.

In the early 1960s, Mackaness established that after inoculation of mice, *L. monocytogenes* can survive and multiply within macrophages (37). Since discovery of this key phenomenon in the pathogenesis of *L. monocytogenes* infection, experimental murine listeriosis has been extensively characterized and used as a model to study immune responses to intracellular pathogens. Until recently, little was known about the virulence factors of *L. monocytogenes*, although this facultative intracellular bacterium possesses two easily recognizable phenotypes closely associated with virulence: hemolytic activity (1) and egg yolk agar opacification (53), a reaction revealing lecithinase activity (12, 30).

The toxin responsible for the hemolytic activity is a thiol-activated cytolysin named listeriolysin O (LLO), which has been purified and characterized (15). Its structural gene, *hly*, has been cloned and sequenced (42). Genetic experiments have demonstrated that LLO is an essential virulence factor, since *hly* mutants are avirulent. The toxin is necessary for intracellular multiplication of the bacterium in murine macrophages, as well as in epithelial cells (for a review, see reference 7).

The second easily recognizable phenotype, egg yolk agar opacification, first described in 1962 (12), was attributed in 1975 (30) to a secreted phospholipase C (PLC) (18, 23, 24, 26, 47, 60, 70). This PLC was shown to be active on phosphatidylcholine (lecithin), hence the name lecithinase (30). It has been purified from culture supernatants of *L. monocytogenes* EGD (16) and is a protein of 29 kDa that is active not only on phosphatidylcholine (PC) but also on phosphatidylserine, phosphatidylethanolamine, and weakly on sphingomyelin.

Recently, *plcA* (*pic*), a gene that codes for a different *L. monocytogenes* phospholipase, has been cloned and sequenced (4, 32, 39). This is a phosphatidylinositol-specific PLC that is able to cleave glycosyl-phosphatidylinositol-anchored proteins (39). Although *plcA* mutants are affected in virulence, the role of phosphatidylinositol-specific PLC in virulence is unclear, since the mutations have a polar effect on a downstream gene, *prfA*, which encodes a pleiotropic activator of virulence genes (33, 40).

Following the pioneering electron microscopic observations of Racz et al. on infected tissues in the early 1970s (51, 52), development of in vitro models of infection using

* Corresponding author.
† Present address: Departamento Patología Animal I, Facultad de Veterinaria (UCM), 28040 Madrid, Spain.

various cell lines (14, 50) has led to a precise description of the cell biology of the infectious process (9, 20, 28, 46, 64, 65), in particular, the phenomenon of cell-to-cell spread. The first step of the infection is uptake of bacteria by the host cell. The bacteria are able to actively induce their own phagocytosis by nonprofessional phagocytes, e.g., epithelial cells. They then lyse the phagosomal membrane and escape into the cytoplasm. Once in the cytoplasm, the bacteria can grow and move by an unknown mechanism involving polymerization of cellular actin. Some bacteria become incorporated into long cytoplasmic protrusions extending from the periphery of an infected cell into neighboring cells. These protrusions, harboring at the tip a bacterium followed by an actin tail, are taken up by neighboring cells. Subsequently, bacteria are seen in vacuoles surrounded by two membranes, the inner one stemming from the plasma membrane of the first host cell and the outer one originating from the newly infected cell. Both membranes have to be lysed to allow the bacterium to enter the cytoplasm of the new host cell, where replication and a new cycle of further spread to new host cells can take place.

The first step of the infectious cycle, entry, is mediated by internalin, a 744-amino-acid, repeat-containing protein probably located in the bacterial membrane (13). The second step, escape from the phagosome by lysis of the phagosomal membrane, is mediated by LLO. This molecule has been shown to be necessary and sufficient for phagosomal lysis (3, 14). Recently, it has been proposed that phospholipases may also play important roles in the infectious process (4, 39, 46, 63), in particular, lecithinase at the step of lysis of the two-membrane vacuoles surrounding bacteria after cell-to-cell spread (63).

We investigated the role of lecithinase in cell infection by studying lecithinase-deficient mutants obtained by transposon mutagenesis. In one mutant (41), the transposon has inserted into *mpl* (*prtA*), a gene that is located immediately downstream from *hly* and encodes a putative metalloprotease. This gene is transcribed on a 5.7-kb RNA, suggesting that the lecithinase deficiency of the mutant was due to a polar effect on downstream genes, in particular, on the gene that encodes lecithinase. This hypothesis led us to clone and sequence the downstream region. We identified the lecithinase gene of *L. monocytogenes* LO28 as the third gene of an operon and present results that suggest that the lecithinase is involved in cell-to-cell spread, a key phenomenon of *L. monocytogenes* pathogenicity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. monocytogenes* LO28, a clinical isolate (68), was grown at 37°C with aeration in brain heart infusion (Difco Laboratories, Detroit, Mich.) broth or on brain heart infusion agar plates. *Escherichia coli* MC1061 (6) or TG1 (5) was grown in LB medium (45) containing 25 to 100 μg of ampicillin per ml (liquid or solid medium, respectively) when harboring pBR322 or derivatives. For infection of cell cultures, bacteria were grown for 15 h at 37°C in brain heart infusion to a density of about 2.5×10^9 CFU/ml and washed once in phosphate-buffered saline. CFUs were determined by plating bacteria, at appropriate dilutions in phosphate-buffered saline, onto brain heart infusion agar plates and counting colonies the next day.

DNA techniques, enzymes, and reagents. All of the DNA techniques used have already been described (39–41, 43, 44).

DNA sequencing. The 5,648-bp *Hin*dIII-*Eco*RI *L. monocytogenes* DNA fragment cloned into pBR322 (plasmid pLmo2) was purified and cut by *Rsa*I or *Hae*III and ligated to M13 mp8 restricted by *Sma*I. *E. coli* TG1 was transformed with ligation mixtures and plated on LB plates with 4 ml of H top agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside (44). Single-stranded DNAs were prepared from white plaques, as previously described (44), and sequenced with the universal primer. The DNA sequences obtained by this technique were extended by directly sequencing plasmid pLmo2 with oligonucleotide primers (18- to 21-mer) derived from the sequence. The DNA sequence was determined on both strands of the DNA by the dideoxy-chain termination method of Sanger et al. (57) by using [^{35}S]dATP (800 Ci/mmol) and Sequenase kit no. 2 from USB.

Computer analysis of sequences. Nucleic acid sequences were analyzed on Apple MacIntosh computers with the DNA-Strider 1.1 (38) program and on a Data General MV10000 computer at the Unité d'Informatique Scientifique of the Institute Pasteur. Data base searches for amino acid sequence similarities were done by using FASTA (49) on a translated gene bank (Genpept; release no. 64.3) and the Swiss-Prot data bank (release no. 17.0) or by using FASTP (35) to search in the bacterial subdivision of a translated gene bank (PGtrans; release no. 63.0).

Promoter mapping. The transcription initiation site of *actA* was determined by primer extension analysis, as previously described (41, 43), by using oligonucleotide GGTTAATCG TAATGCAATTGG, which is present in the coding strand of *actA* (positions 297 to 277 in Fig. 2).

Southern blot analysis. Chromosomal DNA was prepared as previously described (41) from *L. monocytogenes* type strain CIP 82110^T, *L. monocytogenes* LO28 (68), *L. monocytogenes* EGD, *L. ivanovii* type strain CIP 7842^T, *L. ivanovii* SLCC 4121, *L. ivanovii* CLIP 257, *L. seeligeri* type strain CLIP 12513^T, *L. seeligeri* SLCC 3503, *L. seeligeri* CLIP 9529, *L. innocua* type strain CIP 8011^T, *L. innocua* CLIP 11262, *L. welshimeri* type strain CLIP 12514^T, *L. welshimeri* SLCC 5328, and *L. murrayi* ATCC 25401 and restricted with *Hin*dIII or *Hin*dIII-*Eco*RI. Southern blots under low-stringency hybridization conditions were performed as previously described (19). Probes were prepared by polymerase chain reaction on plasmid pLmo2 and purified with the Geneclean kit (BIO 101 Inc., La Jolla, Calif.). The probe for *actA* was made with oligonucleotides GGG ATTAAACAGATTTATGC (positions 219 to 240 in Fig. 2) and TCTGTGTTTTTAATTATTTTTC (positions 2156 to 2133 in Fig. 2). Probes for *plcB* were made with oligonucleotides GCTATTGGCGTGTTCTCT (positions 2085 to 2102 in Fig. 2), ACCCGACAATACTGACG (positions 2348 to 2356 in Fig. 2), and AGTCTAGCTCCAGTAGGT (positions 2989 to 2972 in Fig. 2). The probe for open reading frame X (ORFX) to ORFZ was made with oligonucleotides GTGT-TAGTAATCCGCGTTA (positions 3160 to 3178 in Fig. 2) and AGCCTTGGGATTCTAGA (positions 4023 to 4007 in Fig. 2). The probe for ORFB was made with oligonucleotides TTCGATGACAACAGCTGTAC (positions 4112 to 4132 in Fig. 2) and TTCACCAAATATTGAAGGCAA (positions 4415 to 4394 in Fig. 2). The probe for lactate dehydrogenase (*ldh*) was made with oligonucleotides CGGCGTTGCTGCA GCTCT (positions 5490 to 5474 in Fig. 2) and CATTGC GTCTAGAACT (positions 5253 to 5271 in Fig. 2).

Cell culture. Mouse macrophagelike cell line J774 (ECACC 85011428) and murine 3T3 fibroblasts (ECACC 88031146) were propagated in DMEM (GIBCO) supplemented with 10% fetal calf serum (decomplemented for 30

min at 56°C) (Boehringer). Mouse fibroblast line L2 (54) was grown in DMEM containing 5% fetal calf serum. All cell lines were maintained with no antibiotics.

Plaque assays on monolayers of L2 and 3T3 fibroblasts. For plaque formation assays on L2 cells, we used the method of Sun et al. (63) with minor modifications. Monolayers in tissue culture petri dishes (60 by 15 mm; Corning) were infected with 1.5 to 5 μl of a 10^{-3} dilution of a washed bacterial overnight culture. The agarose overlay contained 0.75% agarose, and readout of plaques was done at day 5 or 6 of infection. Plaque assays on 3T3 cells were performed as previously described (28), except that infections were done at various inoculum concentrations: 5 and 25 μl of bacterial 2-h subcultures, either undiluted or diluted 10-fold in phosphate-buffered saline. Readout of plaques was clearest at 3 or 4 days of infection.

Transmission electron microscopy of *L. monocytogenes*-infected macrophages. For electron microscopy, 2.5×10^6 J774 macrophages in 2 ml of growth medium were seeded into tissue culture petri dishes (35 by 10 mm; Corning) 24 h prior to use. Infection was performed essentially as described by Tilney and Portnoy (65). For 90 min of infection, 100 μl of a 10-fold-diluted, washed bacterial overnight culture was added to the monolayers; for 4 h of infection, 20 μl was used; and for 5.5 h of infection, 30 μl was used. This corresponds to a multiplicity of infection of approximately 7.5 bacteria per macrophage for the 90-min infection period, 1.5 for the 4-h infection period, and 2 for the 5.5-h infection period, respectively. After an initial infection period of 30 min, monolayers were washed three times at 37°C with phosphate-buffered saline and overlaid with 2 ml of prewarmed growth medium containing 5 μg of gentamicin per ml. At this concentration, gentamicin kills extracellular but not intracellular bacteria and thus does not affect intracellular growth and cell-to-cell spread of *L. monocytogenes* (14, 50, 63). In situ fixation, staining, embedding, and further processing were done as previously described (65). Sections of infected cells and noninfected controls were examined by using a Philips CM12 electron microscope. For quantitative analysis, fields of sections of infected cells were selected randomly and all bacteria present inside or outside of vacuoles were counted.

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 2 has the GenBank accession no. M82881.

RESULTS

In this paper, the gene nomenclature corresponds to that recently adopted (49a): *hly* for *hlyA* (42) and *lisA* (10); *plcA* for ORFU (43), *pic* (32), and *plcA* (4, 39); *prfA* for *prfA* (33, 40); *mpl* for ORFD (43), *prtA* (41), and *mpl* (11); *actA* for *prtB* (40); and *plcB* for *prtC* (40).

Cloning and sequencing of the *Hin*dIII-*Eco*RI fragment located downstream from *mpl*. In a previous report (41), we demonstrated that (i) the region located immediately downstream from *hly* encodes a 1,533-bp ORF named *mpl* whose deduced amino acid sequence is homologous to metalloproteases of bacilli, other bacteria, and parasites and (ii) that *mpl* is transcribed on a 5.7-kb RNA. This suggested that *mpl* is the first gene of an operon. By use of a 600-bp probe internal to the 2-kb insert of plasmid pLis22 (Fig. 1) which contains the 3′ end of *mpl* and some downstream region, we identified a *Hin*dIII-*Eco*RI fragment of about 6 kb on Southern blots of chromosomal DNA. This fragment was cloned in pBR322 (plasmid pLmo2). Previous attempts to clone it in high-copy-number pUC vectors had led to unstable recombinant plasmids. The complete nucleotide sequence was determined on both strands of the DNA. The pLmo2 insert is 5,648 bp long. Its sequence is given in Fig. 2. It is A+T rich, in accordance with the low G+C content (38%) of *Listeria* DNA (62).

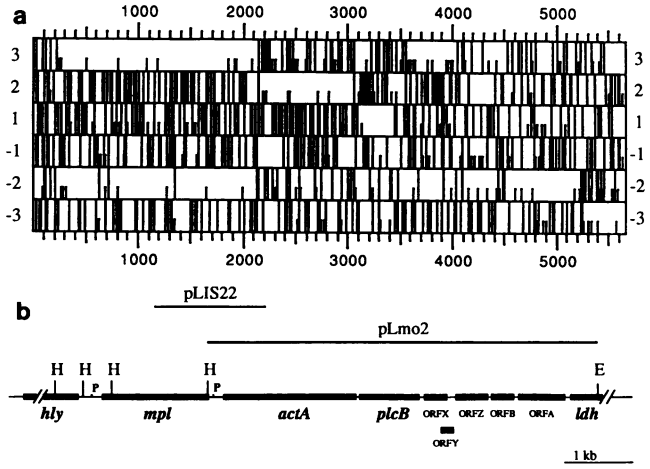

FIG. 1. The lecithinase region of *L. monocytogenes*. (A) ORF map of the 5,648-bp *Hin*dIII-*Eco*RI insert in pLmo2. (B) Schematic representation of the lecithinase operon and its downstream region. The upper lines represent the inserts of plasmids pLis22 and pLmo2. Below, the genetic organization of the region is given. Thick lines correspond to the various genes and ORFs; P corresponds to promoters. Restriction sites are indicated. H, *Hin*dIII; E, *Eco*RI.

Genetic organization of the lecithinase operon and its downstream region. The genetic organization of the lecithinase operon and its downstream region is depicted in Fig. 1. The last 29 nucleotides of the *mpl* gene are present downstream of the *Hin*dIII site of the pLmo2 insert. As previously reported, no palindromic sequence which could act as a transcription termination signal was found downstream from *mpl*. Another ORF was present 199 bp downstream from the stop codon of *mpl* and in the same orientation. This ORF, named *actA*, starts with a GTG codon and is preceded 6 bp upstream by a putative ribosome-binding site, the GGAGG sequence complementary to the 3′-terminal sequence of the *L. monocytogenes* 16S rRNA (36). At 15 bp downstream from the GTG codon, there are three in-frame ATG codons. If the GTG codon is considered the translation start codon, *actA* is 1917 bp long. It ends by an ocher codon and could encode a protein of 639 amino acids.

Four other ORFs were found in the same orientation as *mpl* and *actA*. Thirty-five base pairs downstream from the *actA* stop codon, a 867-bp-long ORF starts with an ATG codon. This ORF, named *plcB*, is preceded by a putative ribosome-binding site, GAGG, and would encode a protein of 289 amino acids. At 50 bp downstream from the *plcB* stop codon, a 321-bp ORF, ORFX, starts with an ATG codon. ORFX would encode a peptide of 107 amino acids. A 134-bp sequence extends between the ORFX stop codon and the start codon of a 459-bp ORF, ORFZ, predicted to encode a 153-amino-acid polypeptide. Both ORFX and ORFZ are preceded by 9 and 5 bp by putative ribosome-binding sites. An additional overlapping ORF of 177 bp spanning the last 80 bp of ORFX and the ORFX-ORFZ intergenic region was detected. This ORF, ORFY, is 177 bp long and would encode a 59-amino-acid peptide; it starts with an ATG codon

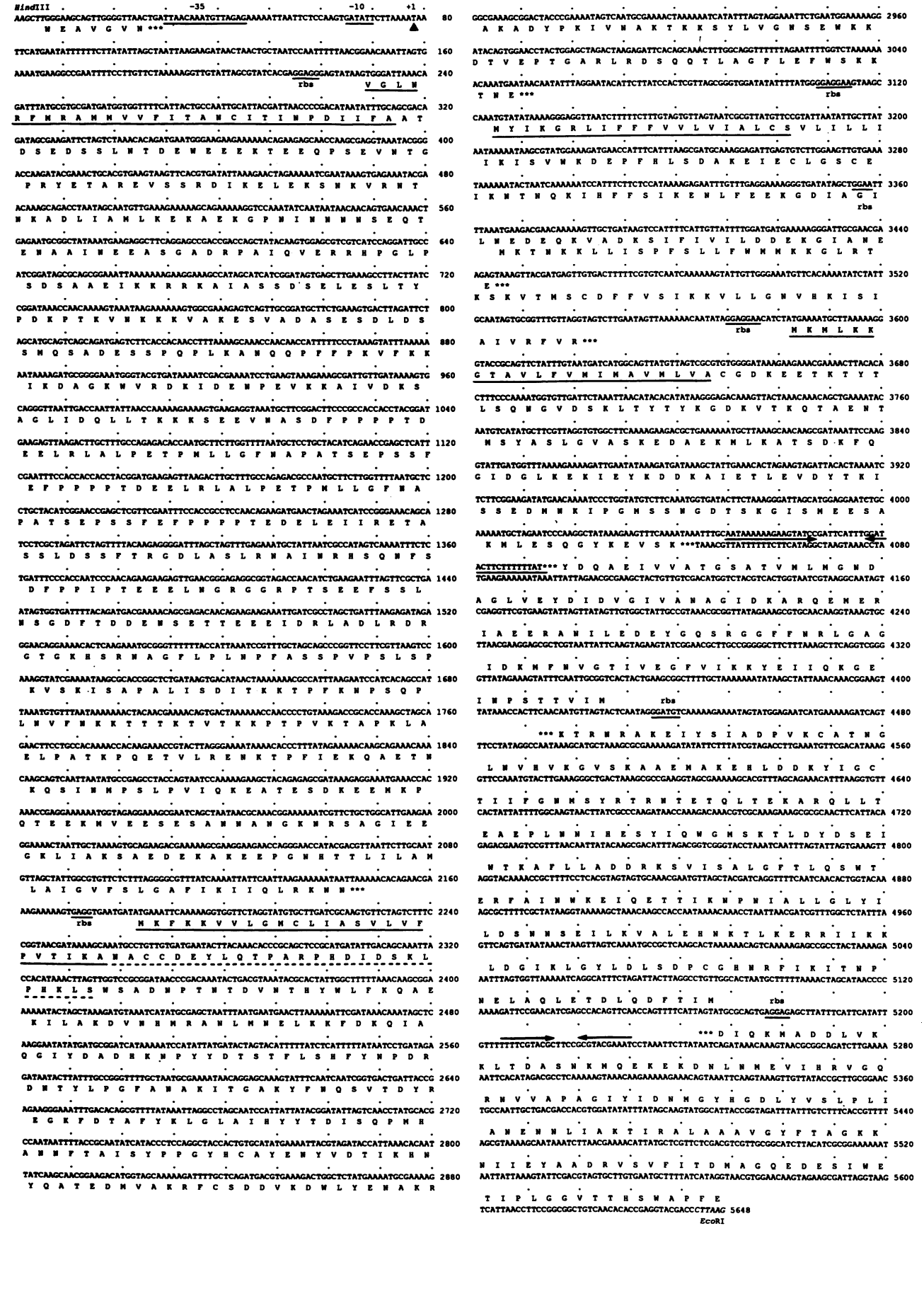
HindIII
-35
-10
+1
rbs
rbs
rbs
rbs
rbs
EcoRI

FIG. 2. Complete nucleotide sequence of the pLmo2 insert. The translation of *actA*, *plcB*, ORFX, ORFZ, ORFY, *ldh*, ORFA, and ORFB is given below or over the nucleotide sequence in the single-letter amino acid code. Note that the sequence of the noncoding strand is given for all ORFs: the 5′ strand up to nucleotide 4093 and the 3′ strand from nucleotide 4044 on. A black triangle indicates the transcriptional start site in front of *actA*; the −10 and −35 regions are indicated. Arrows indicate predicted terminators. rbs indicates putative ribosome-binding sites. The putative signal sequences of *actA*, *plcB*, ORFX, and ORFZ are underlined. The asterisks indicate the stop codons.

preceded 6 bp upstream by a putative ribosome-binding site. Its stop codon is located 37 bp upstream from the ATG of ORFZ. Finally, we found a thermodynamically stable (ΔG = −26.1 kcal [1 cal = 4.184 JJ/mol) palindromic sequence 6 bp downstream from the stop codon of ORFZ. This palindrome is probably the transcription termination signal of the operon. Indeed, the length of the DNA fragment extending from the transcription initiation site of *mpl* up to the stop codon of ORFZ is 5,697 bp long, in agreement with the size of 5.7 kb previously determined for the *mpl* transcript (41).

No further ORFs were found on the same strand of the DNA. On the other strand of the DNA, three ORFs were detected. The first one starts at the *Eco*RI site. It is 405 codons long and would encode 135 amino acids. This ORF, named *ldh*, ends at position 5244 of Fig. 2 and is followed 15 bp downstream from its stop codon by a palindromic region which could be a transcription terminator. ORFA, a 675-bp ORF which would encode a 225-amino-acid protein, starts 79 bp downstream from the *ldh* stop codon. Finally, 67 bp from the ORFA stop codon starts ORFB, which is 336 bp long and could encode a 112-amino-acid polypeptide. Its stop codon is located just after the 3′ end of the terminator of the large operon.

***plcB* encodes the lecithinase of *L. monocytogenes*.** On the basis of the lecithinase-negative phenotype of an *mpl* transposon insertion mutant and by assuming the possibility of a polar effect of the transposon insertion, we anticipated that the lecithinase gene of *L. monocytogenes* might be located downstream from *mpl* (41). This is indeed the case. Several lines of evidence have led to the identification of *plcB* as the lecithinase gene of *L. monocytogenes*. (i) Lecithinase activity was detected in extracts of *E. coli* harboring plasmid pLmo2, as shown in Fig. 3. (ii) The sequence of the *plcB*-encoded protein contains, at position 94, the sequence Tyr-Phe-Asn-Gln-Ser-Val-Thr-Asp-Tyr, a tryptic nonapeptide identified and sequenced from the lecithinase purified from *L. monocytogenes* supernatants (16). (iii) *plcB* encodes a 289-amino-acid protein similar to the PC-preferring PLC (PC-PLC) of *Bacillus cereus* (17, 25) and to the first two-thirds of the alpha-toxin of *Clostridium perfringens* (34, 56, 66, 67). The homologies with the *B. cereus* and *C. perfringens* enzymes (38.7% identity on a 253-amino-acid overlap and 22% identity on a 223-amino-acid overlap, respectively) lie all along the sequence (Fig. 4). (iv) Disruption of *plcB* abolished lecithinase activity. The lecithinase gene was interrupted after codon 170 (residue 119 in Fig. 4) by insertion of a thermosensitive vector containing a part of *plcB* (26b). The *plcB* mutant displayed no lecithinase activity on egg yolk agar plates, whereas an ORFX-ORFY mutant obtained in the same way, interrupting ORFX after codon 102 and ORFY after codon 22, had a lecithinase phenotype indistinguishable from the wild type (Fig. 5).

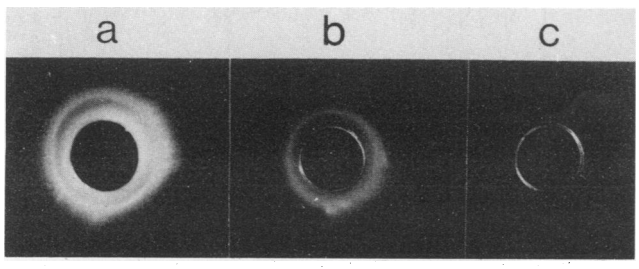

FIG. 3. Detection of lecithinase activity on egg yolk agar. Wells of an egg yolk agar plate were filled with either a concentrated culture supernatant of *L. monocytogenes* (a) or a supernatant of sonicated *E. coli* MC1061 cells harboring plasmid pLmo2 (b) or pBR322 (c).

***actA* encodes a protein with features of surface proteins of gram-positive bacteria.** *actA* encodes a putative protein of 639 amino acids (Fig. 2). This protein is rich in glutamic acid (11.4%), lysine (9.1%), serine (9.5%), and proline (8.9%). The hydrophobicity plot suggests that it has a signal sequence and a membrane anchor (Fig. 6a). Indeed, its N terminus presents all of the characteristic features of a signal peptide with a predicted peptidase cleavage site between Ala-29 and Ala-30 (69). The predicted amino acid sequence of *actA* revealed a region of repeats (Fig. 6b), a feature frequently found in surface proteins of gram-positive bacteria, including *Listeria* spp. (13). It contains two proline-rich repeats of 35 nearly identical amino acids. Two other, albeit partial, repeats found just after the first two repeats contain the first 10 amino acids of the motif. Another partial repeat was found further downstream in the sequence (Fig. 6b). At the DNA level, these regions are highly conserved, with changes occurring only at the third positions of codons. Finally, the tripeptide Arg-Gly-Asp, a sequence able to interact with integrins (55), was detected at position 360 in the *actA*-encoded protein. No strong homologies to other proteins were found in data bank searches.

Identification of the putative lactate dehydrogenase gene of *L. monocytogenes*. The three last ORFs of the lecithinase operon, ORFX, ORFY, and ORFZ, encode proteins with unknown functions. ORFX would encode a 107-amino-acid protein. Its N-terminal sequence has all of the features ascribed to signal sequences (69). The predicted ORFX gene product is relatively rich in cysteine (three residues). The ORFY-encoded polypeptide is 59 amino acids long and is rich in lysine (17%). The protein encoded by ORFZ is 153 amino acids long. It also presents a putative signal sequence and is also rich in lysine (15%). The ORFX, ORFY, and ORFZ, gene products had no sequence similarity to known proteins.

Three ORFs in the orientation opposite to that of the lecithinase-containing operon were identified. The first one starts at the *Eco*RI site and is 395 bp long. The deduced amino acid sequence of this 134-amino-acid ORF is similar (more than 50% conserved residues) to the C-terminal part of the lactate dehydrogenases of several bacilli (2, 48, 71), *Lactobacillus casei* (26a), and *Lactococcus lactis* (9a), as well as to eucaryotic lactate dehydrogenases (21, 58). We therefore named this gene *ldh*. It probably represents the lactacte dehydrogenase gene of *L. monocytogenes*. The proteins encoded by ORFA and ORFB had no homologies to known proteins.

FIG. 4. Amino acid sequence alignments of the PC-PLC of *L. monocytogenes*, *B. cereus* (17, 25), and *C. perfringens* (34, 56, 66, 67). Identical amino acids are boxed. The numbering is according to the amino acid sequence of the mature protein of *B. cereus* (see Discussion and reference 25). The asterisks indicate amino acids involved in binding to the Zn^{2+} atoms in the *B. cereus* enzyme (22).

A second promoter is present upstream from *actA*. In a previous study, the transcriptional start site of *mpl* had been mapped 148 bp upstream from the putative *mpl* translation initiation codon ATG (43) and a 5.7-kb-long transcript had been identified (41). We suspected the presence of a promoter upstream of *actA*, because mutations in *mpl* only partially impaired lecithinase expression. Primer extension analysis led to the identification of another transcription start site located 158 bp upstream from the *actA* initiation codon (Fig. 7), in the *mpl-actA* intergenic region. As shown in Fig. 7, this promoter has homologies with the *hly*, *plcA*, and *mpl* promoters. At position −35, a 14-bp palindrome is present in the promoters controlled by regulatory gene *prfA* (40) but is not present in the promotors of genes that are not regulated by *prfA*.

Part of the lecithinase operon is specific for *L. monocytogenes*. By use of an *mpl*-specific probe in Southern hybridization experiments performed previously under low-stringency conditions, no signal had been detected in any of the six other species of the genus *Listeria* (19, 31). The same type of analysis was performed in this study (see Materials and Methods). With an *actA* probe, no signal was detected in *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, or *L. murrayi*. With the *plcB* probe, hybridizing bands were detected in all three *L. ivanovii* strains (*Hin*dIII fragments of approximately 650 bp) and in all three *L. seeligeri* strains (CLIP 12513 and SLCC 3503, *Hin*dIII fragments of approximately 2.5 kb; CLIP 9529, *Hin*dIII fragment of approximately 4.5 kb). With a probe spanning ORFX, ORFY, and ORFZ, a signal was detected in both *L. innocua* strains. In

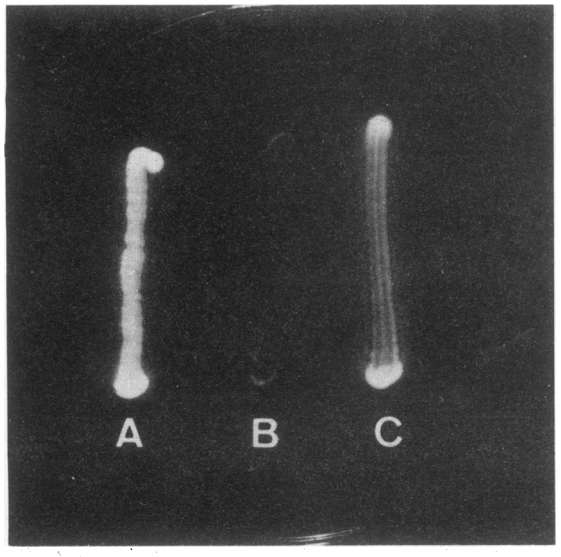

FIG. 5. Detection of lecithinase activity on egg yolk agar plates. A, wild-type strain LO28; B, the *plcB* mutant; C, the ORFX-ORFY mutant (see Results).

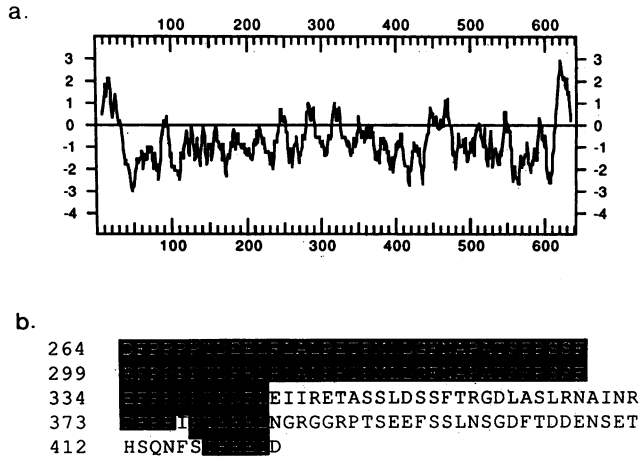

FIG. 6. Features of the predicted protein encoded by *actA*. (a) Hydrophobicity plot (29). (b) Alignment of the internal repeats. Amino acid positions are indicated.

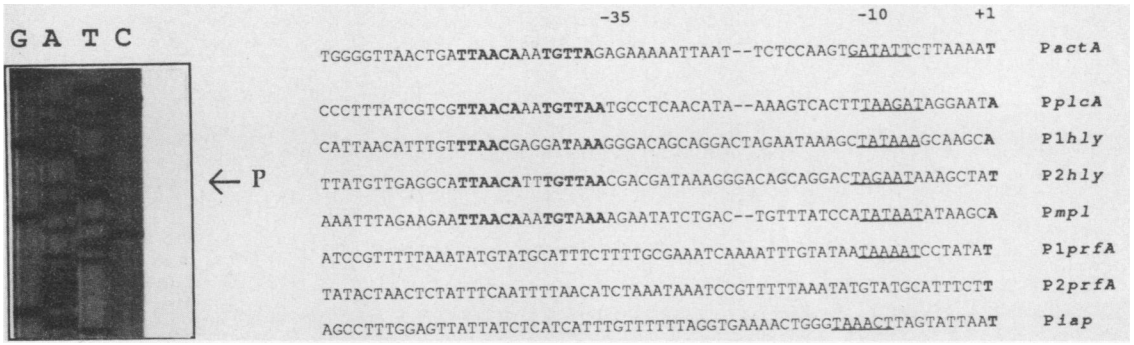

FIG. 7. Promoter comparison. The *actA* promoter (P) was identified by primer extension analysis as shown on the left. The sequence of the promoter located upstream from *actA* was compared and aligned with the promoters of *mpl* (43), *hly* (43), *plcA* (43) *prfA* (40), and *iap* (27) as shown on the right. The conserved palindromic region located in the −35 region of each promoter controlled by the *prfA* gene product (43) and the identified +1 nucleotides are in boldface.

contrast to this, signals were detected in all six species of the genus *Listeria* with an ORFB (Fig. 8)- or an *ldh*-specific probe.

***plcB* mutants are affected in cell-to-cell spread.** To investigate at which step in the cycle of cell infection the lecithinase acts and to determine whether this enzyme plays a role in cell infection by direct cell-to-cell spread, we examined the plaque formation capacity of the *plcB* mutant on monolayers of L2 or 3T3 fibroblasts. The plaque assay reflects the ability of *L. monocytogenes* to multiply intracellularly and to spread within fibroblast monolayers covered with an agarose overlay containing gentamicin at a concentration lethal for extracellular but not intracellular bacteria (20). After several days, zones of dead cells, destroyed by bacterial infection, were macroscopically visible as "plaques." As can be seen in Fig. 9, on monolayers of L2 fibroblasts, the *plcB* mutant formed smaller plaques than the wild type, in contrast to the ORFX-ORFY mutant, which formed plaques of normal size. This suggests that the small-plaque phenotype was due to lack of expression of *plcB*.

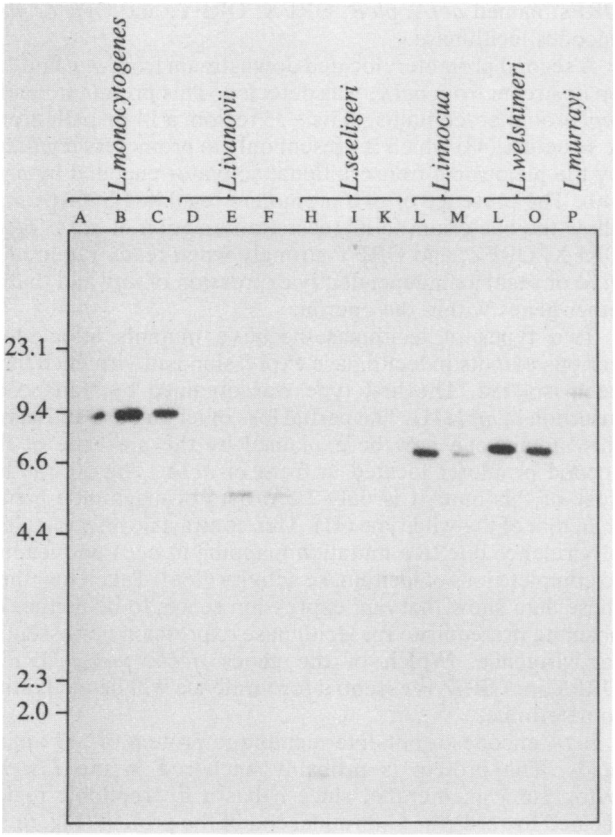

FIG. 8. Southern blot of *Listeria* species with the ORFB probe. Chromosomal DNAs were digested with *Hin*dIII and hybridized under low-stringency conditions with an ORFB probe (see Materials and Methods). Lanes: 1, *L. monocytogenes* type strain CIP 82110^T; 2, *L. monocytogenes* LO28 (68); 3, *L. monocytogenes* EGD; 4, *L. ivanovii* type strain CIP 7842^T; 5, *L. ivanovii* SLC 4121; 6, *L. ivanovii* CLIP 257; 7, *L. seeligeri* type strain CLIP 12513^T; 8, *L. seeligeri* SLCC 3503; 9, *L. seeligeri* CLIP 9529; 10, *L. innocua* type strain CIP 8011^T; 11, *L. innocua* CLIP 11262; 12, *L. welshimeri* type strain CLIP 12514^T; 13, *L. welshimeri* SLCC 5328; 14, *L. murrayi* ATCC 25401.

Infected cells were also observed by electron microscopy. We infected monolayers of J774 macrophages with either the *plcB* mutant or wild-type bacteria and, as a control, with a previously isolated isogenic *hly* mutant (8). After initial infection for 0.5 h, the monolayers were incubated in gentamicin-containing medium for various periods. (The presence of gentamicin in the growth medium prevents new entry of bacteria from the extracellular space and ensures that the infection can proceed only within the monolayers [20].) The infected cells were fixed and observed. Whereas the *hly* mutant, in agreement with previously published results (14), was unable to escape from the phagosome of infected macrophages (Table 1), no difference between wild-type bacteria and the *plcB* mutant was observed at 90 min and at 4 h of infection. Both types of bacteria effectively lysed the phagosomal membranes and multiplied within the cytoplasm. In addition, no difference in accumulation of electron-dense material around the bacteria, presumably actin (64, 65), or in actin tail formation could be observed.

In contrast to this, a statistically significant difference between the wild type and the *plcB* mutant was observed after 5.5 h of infection: 50% of the bacteria were lying free in the cytoplasm of newly infected cells in the case of the wild-type, whereas the *plcB* mutants accumulated inside two membrane vacuoles (Fig. 10a and Table 1). This type of vacuole formed after cell-to-cell spread is easily recognized, since the bacteria are surrounded by two cytoplasmic membranes: an inner one, stemming from the previous host cell and an outer one contributed by the newly infected host cell (Fig. 10 b to d). As expected, nonhemolytic *hly* mutants were present only in low numbers in sections of J774 cells,

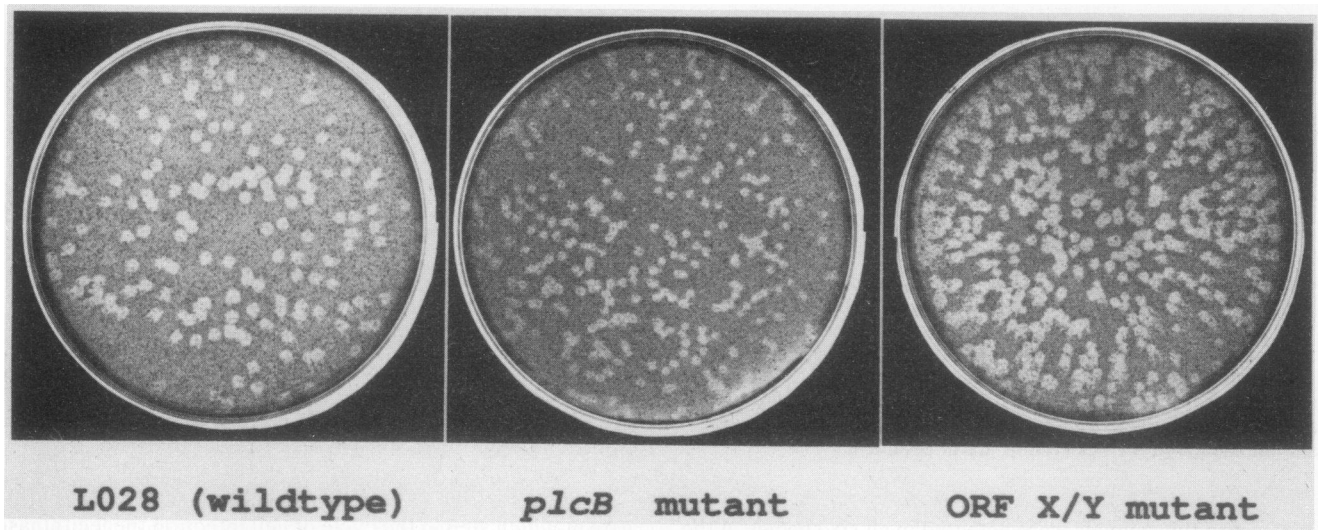

FIG. 9. Plaque formation by *L. monocytogenes* on L2 fibroblasts.

because they could not escape from the phagosome into the cytoplasm and thus did not multiply (14).

Taken together, these results suggest that lecithinase is one of the factors that contribute to efficient lysis of the two membranes that enclose the bacteria after direct cell-to-cell spread. We found no evidence that lecithinase contributes to lysis of the phagosome after primary uptake of bacteria from the extracellular medium.

DISCUSSION

The lecithinase gene of *L. monocytogenes* is the third gene of the operon lying downstream from *hly*. The first gene of this operon was initially called ORFD (43) and recently renamed *prtA* (41) and *mpl* (11) when its sequence was determined and shown to encode a protein similar to bacterial metalloproteases. In addition, it was shown that *mpl*-specific transcripts are 5.7 kb long (41), thus predicting the presence of an operon. We have now completed the sequencing of this operon and shown that it contains five other ORFs, named *actA*, *plcB*, ORFX, ORFY, and ORFZ. *plcB* encodes lecithinase.

TABLE 1. Electron microscopic study of accumulation of *plcB* mutants in double-membrane vacuoles after direct cell-to-cell spread in J774 macrophages at 5.5 h postinfection with *L. monocytogenes*

Strain	% Bacteria observed[a]		
	Surrounded by membranes		Free in the cytoplasm
	Completely	Partially	
Wild type	47 (184)	3 (11)	50 (197)
plcB mutant[b]			
Expt 1	67 (211)	0 (1)	33 (103)
Expt 2	69 (438)	0 (1)	31 (199)
hly mutant (control)			
Expt 1	92 (11)	8 (1)	0 (0)
Expt 2	78 (7)	22 (2)	0 (0)

[a] Absolute numbers are in parentheses.

[b] The statistical significance of the difference between wild-type and *plcB* mutant bacteria (% of bacteria surrounded by membranes) was calculated with the chi-square test. The difference was highly significant at $P < 10^{-6}$.

A second promoter, located downstream from *mpl* and 158 bp upstream from *actA*, was detected. This promoter, as the *mpl* promoter, contains, in its −35 region, a 14-bp palindromic structure (43) which is present only in promoters regulated by the pleiotropic transcriptional activator encoded by *prfA* (40). The presence of two promoters regulated by *prfA* may allow the bacterium to increase transcription of *actA*, *plcB*, ORFX, ORFZ, and ORFY strongly when really required in vivo or regulate independently expression of *mpl* and that of other genes within the operon.

Two types of lecithinase-negative mutants affected to various extents in lecithinase expression and virulence have been isolated. The first type was obtained by transposon insertion in *mpl* (41). The partial loss of lecithinase activity in this mutant can now be explained by the presence of the second promoter located in front of *actA*. The 50% lethal dose of this mutant is only 1.5 orders of magnitude higher than that of the wild type (41). This contrasts with a total loss of virulence due to a mutation mapping in *actA* and leading to complete loss of lecithinase activity (26a). Taken together, these data show that *mpl* expression seems to be neither an absolute prerequisite for lecithinase expression nor essential for virulence. Which of the genes *actA*, *plcB*, ORFX, ORFY, or ORFZ is essential for virulence will be interesting to determine.

actA encodes a putative membrane protein of 639 amino acids. The protein is probably anchored in the *Listeria* cytoplasmic membrane, since it has a hydrophobic region located towards the C-terminal end of the protein (Fig. 6). In gram-positive bacteria, membrane anchor regions are often preceded by the consensus sequence Leu-Pro-X-Thr-Gly-Glu (LPXTGE) (11b, 13) and it has been proposed that this motif is responsible for anchoring of surface proteins in the cytoplasmic membrane (11a). In the *actA* product, a sequence close to the LPXTGE consensus, namely, Leu-Pro-Ala-Thr-Lys (LPATK), is present at amino acid position 513 but is not followed by hydrophobic residues. The *actA* protein presents a feature characteristic of surface proteins of gram-positive bacteria, i.e., a region of internal amino

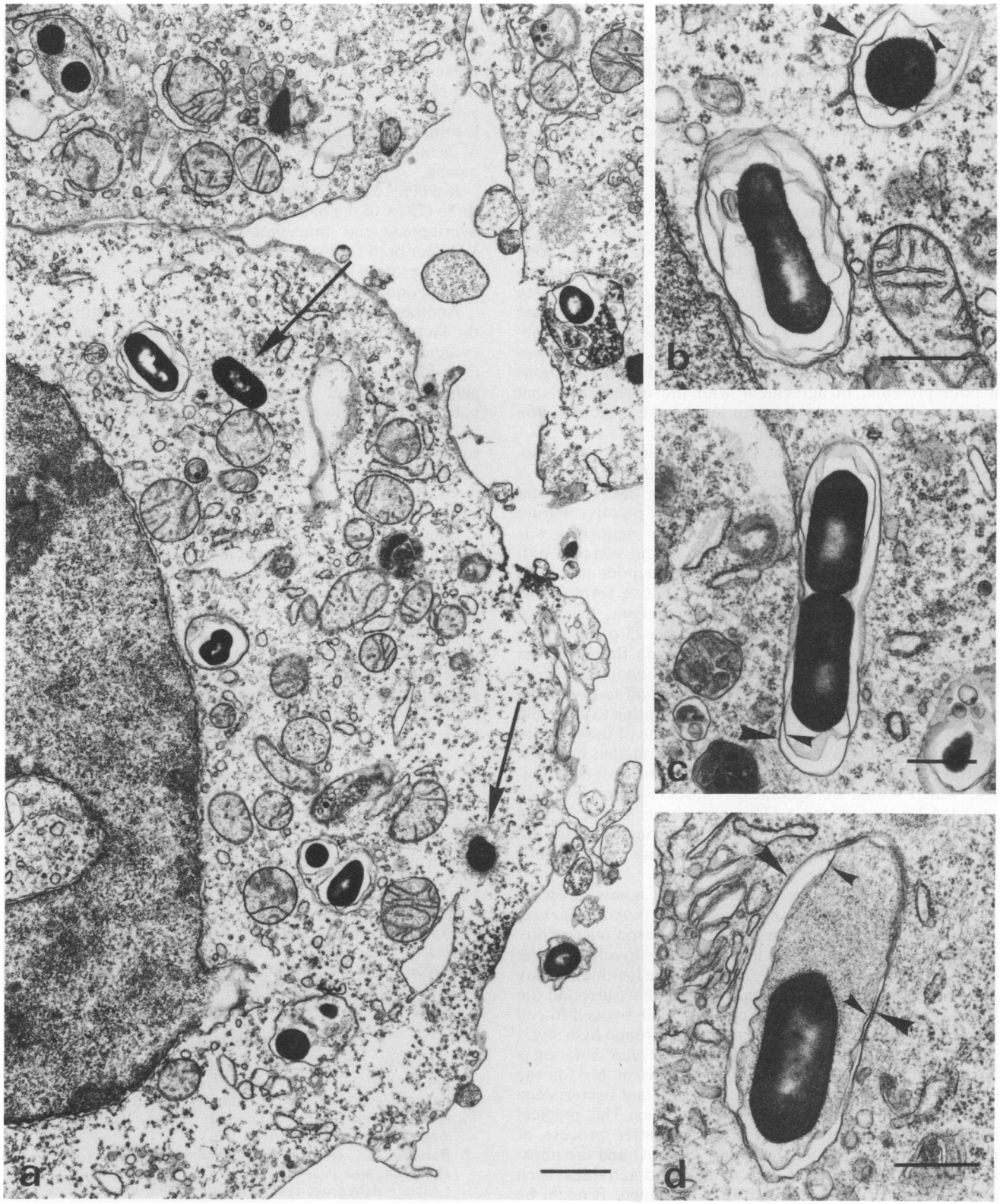

FIG. 10. Thin sections of J774 macrophages infected with *plcB* mutant *L. monocytogenes*. Macrophages were infected for 30 min with *plcB* mutant bacteria and then incubated for 5.5 h in gentamicin-containing medium. (a) Most bacteria are in vacuoles possessing two membranes. Two bacteria are lying free in the cytoplasm (arrows). One is surrounded by filamentous material, presumably actin (magnification, ×6,500). (b and c) Internalized bacteria are surrounded by two cytoplasmic membranes. The inner one stems from the previous host cell (small arrowhead); the outer one stems from the newly invaded host cell (large arrowhead). (d) Internalized bacterium surrounded by residual microfibrillar material, probably representing an actin tail, inside a two-membrane vacuole. The small and large arrowheads are as in panels b and c. Bars: a, 2 μm; b to d, 0.5 μm.

acid repeats. The repeats are proline rich and share some homology with repeats found in the fibronectin-binding protein of *Staphylococcus aureus* (61). In the latter protein, the repeats are predicted to mediate attachment to the cell wall. Because of their position within the protein, it seems unlikely that the proline-rich repeats present in the *actA*-encoded protein play a similar role. Recent results indicate that *actA* is involved in the actin polymerization process (26b).

plcB encodes a protein similar to the PC-PLCs of *B. cereus* and *C. perfringens* (17, 25, 34, 56, 66, 67). The PC-PLC of *B. cereus* has been crystallized, and its three-dimensional structure has been solved at a 1.5-Å resolution (22). In its active site, the *B. cereus* PC-PLC contains three Zn^{2+} atoms bound to nine amino acids. These nine amino acids, namely, Trp-1, His-14, Asp-55, His-69, His-118, Asp-122, His-128, His-142, and Glu-146, in the *L. monocytogenes* lecithinase sequence (Fig. 4) are found in identical positions in both proteins, in agreement with the observation that activity of the *L. monocytogenes* lecithinase is increased in the presence of Zn^{2+} ions (16).

In *B. cereus*, the PC-PLC-encoding gene has been sequenced and the deduced amino acid sequence reveals a classical signal peptide of 24 amino acids that ends after Ala-Phe-Ala (25). When the sequence of the putative mature protein postulated from the nucleic acid sequence was compared to the amino acid sequence of the secreted protein, it appeared that a 14-amino-acid propeptide had been cleaved off to give rise to the mature, active form of the protein (25). In the case of *L. monocytogenes*, the signal peptidase cleavage site would be after Ala-25 of the translated ORF, and amino acid alignments with the *B. cereus* protein suggest that in the case of *L. monocytogenes* a 26-amino-acid propeptide could be cleaved off to produce a mature protein of 238 amino acids. It is tempting to correlate this observation to the fact that the first gene of the lecithinase operon encodes a protease whose substrate has not been identified so far. This substrate could be the prophospholipase which would be specifically processed and possibly activated by this protease. This hypothesis would explain detection of two forms of lecithinase, differing by 3 kDa, on immunoblots of *L. monocytogenes* culture supernatants (16).

Our results bring up the identification of a new molecule involved in the intracellular parasitism of *L. monocytogenes*, i.e., the lecithinase. Plaque assays and electron microscopy of infected cells suggest that this enzyme is involved in the cell-to-cell spread of *L. monocytogenes*. The lecithinase may contribute to lysis of the two membranes that surround the bacteria after invasion of a new host cell by cell-to-cell spread by destabilizing these membranes through hydrolysis of phospholipids. However, the effect of the *plcB* mutation is not as stringent as the effect of mutations in *hly* for the escape from the phagosome, since *plcB* mutant bacteria can still escape from the two-membrane vacuoles. This predicts that other factors are involved in the latter process of membrane lysis. Whether these may be LLO and the phosphatidylinositol-specific PLC or unidentified activities, such as phospholipase A, remains to be determined. It might be argued that the truncated lecithinase predicted to be produced by the *plcB* mutants used in this study may retain some of the lecithinase properties, such as a role for the lecithinase in escape from the phagosome. We think that this is unlikely, because the mutation removes four of nine residues thought to be critical for lecithinase activity (see above) and because *plcB* mutants were completely negative for lecithinase activity on egg yolk agar plates. On the other hand, however, we cannot exclude the possibility that the presence of LLO masks a role for lecithinase in escape from the phagosome, since it has been shown that LLO alone is sufficient for escape from the phagosome (3). Whether the lecithinase contributes to lysis of phagosomes in the absence of hemolysis (14, 46, 50) should be approached by other means.

It will also be of interest to investigate the role of the last three ORFs of the operon. The presence of an ORF (ORFY) overlapping the preceding ORF (ORFX) is intriguing. Whether both ORFs are translated or lead to expression of a unique protein by translational frameshift or another mechanism is not known.

Another finding of the present study was identification of the lactate dehydrogenase gene. It is known that *L. monocytogenes* ferments glucose to produce lactate without gas (59). Identification of the structural gene will allow investigation of whether expression of *ldh* mediates a unique pathway of energy production by *L. monocytogenes* inside and outside of the cell. It will also allow assessment of the role of lactate production in the intracellular environment. It is possible that local acidification of the bacterial environment plays a role in the intracellular life of *L. monocytogenes*.

Finally, we identified the end of the virulence region. Genes *mpl* and *actA* are specific for *L. monocytogenes*, in agreement with a role of this part of the genome in virulence. In contrast, a region hybridizing to ORFX, ORFY, and ORFZ was detected in the nonpathogenic species *L. innocua*, the species most closely related to *L. monocytogenes* in the *Listeria* genus, and sequences hybridizing to the *ldh* gene and to ORFB were detected in all species. These data suggest that the virulence region, which includes the genes for LLO and the two phospholipases, ends downstream from *plcB*.

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